Supplementary Materials and Methods

Zebrafish husbandry

All experiments involving zebrafish were done in accordance with the guidelines laid down by the Institutional Animal Care and Use Committee, of Southern Medical University. Zebrafish adults and embryos were maintained as described previously. The developmental stages were defined according to morphologic criteria. The AB strain, *Tg(cd41:eGFP)*, and *Tg(coronin1a:eGFP)* lines were utilized.

Mutagenesis

TALEN-based gene-targeting was used to target the *mpl* gene in zebrafish, according to a previously published method. Based on sequence information for zebrafish *mpl* (NM_001003467), the target region was located in the third exon of the *mpl* gene locus (left TALEN: 5′-AGAAGACTTCACTTGCT-3′, and right TALEN: 5′-CATAGGACTTTTCAAC-3′). The TALEN plasmids were ordered from Viewsolid Biotech (Beijing, China). The spacer between the recognition arms was a 14-bp fragment with a SfaNI site, which could be efficiently used to test for mutation within the spacer region. Zebrafish embryos were injected with TALEN mRNA at the one-cell stage and genome editing events were analyzed. F0 founders were identified by tail genomic DNA amplification and SfaNI digestion. Heterozygous F1 offspring were sequenced and kept, and F2 homozygous mutants were screened from heterozygous F1-intercrossed progenies, and the wild-type (WT hereafter) from the same mating were kept for control.
Microscopy and imaging

Live specimens were anesthetized with tricaine in embryo medium and observed in depression slides. Fluorescent movies were captured with an Olympus DP71 microscope (Olympus Corporation, Tokyo, Japan) as described previously. Whole-mount or magnified images were taken using a Zeiss AxioCam HRc camera mounted on a Zeiss Discovery V20 microscope (Carl Zeiss AG, Oberkochen, Germany). Fluorescent images were captured with an Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan). For calculating $cd41:GFP^{\text{high}}$ cells in zebrafish larvae, $Tg(cd41:GFP);mpl^{\text{msu3+/}-}$ intercrossed progenies were first performed with anti-GFP antibody staining. Then stained samples were captured images by setting the confocal parameter as pinhole size 35 μm and 488 laser Gain 700 to filter most of $cd41:GFP^{\text{low}}$ signals as reported with some modification, and the fluorescent signals (considered as $cd41:GFP^{\text{high}}$ thrombocytes) were counted.

For counting AGM GFP$^+$ cells, $Tg(cd41:eGFP)$ or $Tg(mpl:eGFP)$ background embryos were raised in the same condition. The harvested staged embryos were mounted individually in 3% methylcellulose and observed under the fluorescent microscope, the GFP$^+$ cells between the 6th somite and the 15th somite (assumed AGM region) were calculated and quantified.

Quantitative polymerase chain reaction

All assays were performed in duplicate. The relative quantity of gene expression was calculated by the $2^{(-\Delta\Delta Ct)}$ method with normalization to the level of Danio rerio actin,
beta 1 (β-actin). Primers used for quantitative polymerase chain reaction (qPCR) are shown in Supplementary Table 1.

Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdU) (Sigma-Aldrich, St. Louis., MO, USA) labeling was performed as described. For GFP and BrdU staining, Tg(cd41:eGFP);WT or Tg(cd41:eGFP);mpl^smu-1 embryos were collected at the appropriate stage and fixed in 4% paraformaldehyde. For BrdU labeled adult fish, cells from whole kidney marrow (KM) were collected on slides by cytospin and fixed. These fixed samples were stained with mouse anti-BrdU (1:50, Sigma-Aldrich, St. Louis., MO, USA) and goat anti-GFP (1:200, Abcam, Cambridge, UK), followed by Alexa Fluor donkey anti-mouse-555 (1:400, Invitrogen, California, USA) and Fluor donkey anti-goat-488 (1:400, Invitrogen, California, USA) for fluorescence visualization.

TUNEL assay

TUNEL assay (In Situ Cell Death Detection Kit, TMR red, Roche, Basel, Switzerland) was performed according to the manual, followed by goat anti-GFP (1:200, Abcam, Cambridge, UK) and Alexa Fluor donkey anti-goat-488 (1:400, Invitrogen, California, USA) for fluorescent visualization.

In vitro RNA synthesis and microinjection

Zebrafish full-length 576-bp tpo cDNA was amplified (forward primer-BamHI:
5’-CGGGATCCATGGAAAGCAACGTGACTGG-3’, reverse primer-XhoI: 5’-CCGACTCGAGCTAGCACCATTGGCATCTCT-3’ and cloned into pCS2 vector. *In vitro* transcription was carried out the mMESSAGE mMACHINE® sp6 kit (Ambion, Cambridge, UK). For injection, 500 pg of *tpo* mRNA was injected into embryos at the one-cell stage.

**Modulation of Jak2**

To inhibit Jak2 activity, we soaked 36-hpf embryos in 20 μM TG101209, a JAK2-specific inhibitor which specificity has been verified in zebrafish embryos. To activate jak2a, gain-of-function *jak2a* V581F mRNA was synthesized and injected into embryos (500 pg/embryo) at the one-cell stage. The *jak2a* morpholino was against an intron-exon junction in *jak2a* (5’-CACACACACACTGACAAATATAA-3’) and injected into 1-cell stage embryos to block *jak2a* mRNA splicing as previously described. For rescue experiments, RNA encoding wild-type *jak2a* was generated by in vitro transcription and injected into 1-cell embryos.

**IL-11 treatment**

Recombinant human interleukin-11 (IL-11) (QILU Pharmaceutical Company, Jinan, China) was dissolved in phosphate-buffered saline (6 mg/mL). For injection, 2 nL of IL-11 was injected into embryos at 48 hours post-fertilization through vessels on the yolk sac.
Transplantation

Cell suspensions were prepared from $Tg(cd41:eGFP);WT$ and $Tg(cd41:eGFP);mpl^{imu3}$ fish as previously described. Two days after receiving a sublethal dose of radiation (25 Gy), 0.2 million cells were injected intracardially into the irradiated recipients (6-month-old WT) using a glass capillary needle (World Precision Instruments, Sarasota, FL, USA; 1B100-6). The kidney marrows of transplanted recipients were collected for FACS analysis at 10 days after transplantation. The engraftment of donor cells in recipients were confirmed by detecting GFP$^+$ cells with FACS (by SSC-A and FITC).

Flow cytometry and cytology

Hematopoietic cells isolated from adult KM in WT or mpl$^{imu3}$ fish were washed and resuspended in 0.9 × PBS plus 5% FBS. The cell suspensions were then passed through a 40-μm pore-size filter for flow cytometry analysis based on forward scatter (FSC-A) and side scatter (SSC-A) using a BD FACS Aria 1 flow cytometer (CA, USA). To compare the thrombocyte percentages in peripheral blood (PB) in adult WT and mpl$^{imu3}$ mutants, PB from $Tg(cd41:eGFP)$ and $Tg(cd41:eGFP);mpl^{imu3}$ adult fish were collected and counted based on GFP fluorescence with a benchtop flow cytometry CytoFLEX (Beckman Coulter, Brea, CA, USA), and $cd41:eGFP^{high}$ cell percentages of WT and mpl$^{imu3}$ mutants were compared.

For comparing $cd41$-GFP$^+$ and mpl-GFP$^+$ cells, cell suspensions were prepared from whole zebrafish at 4 days post-fertilization as described. Dissociated cells
from the two reporter lines were subjected to fluorescence-activated cell sorting (FACS) with an imaging flow cytometry, FlowSight from Amnis (Merk Millipore, Seattle, WA, USA). And the cells from the two reporter lines were sorted with MoFlo XDP flow cytometry (Beckman Coulter, Brea, CA, USA) for expression analysis of thrombocyte markers. For morphologic analyses, sorted *cd41-GFP*+ and *mpl-GFP*+ hematopoietic cells were cytocentrifuged at 400 rpm for 3 minutes and subjected to May-Grünwald and Giemsa (Merck, Germany) staining.

**mpl overexpression**

The plasmid of *hsp-mpl-eGFP* was constructed to drive the expression of *mpl* CDS with a heat-shock promoter. The embryos were injected at one-cell stage and heat-shocked at 40°C for 1 hour from 36 to 72 hpf once daily. The embryos with or without GFP fluorescence were separated and fixed for WISH.

**o-Dianisidine staining**

Embryos were stained for 15 min in dark with 0.6 mM *o*-Dianisidine staining solution (Sigma-Aldrich, St. Louis, MO, USA), 0.01 M sodium acetate (pH 4.5), 0.65% H₂O₂ and 40% (vol/vol) ethanol as previously described.
Supplementary References:


Supplementary Figure Legends

Figure S1. Generation of mpl<sup>smu3</sup> zebrafish mutant by TALENs. (A-C) The zebrafish mpl gene structure. Exons are depicted as boxes. The start coding region is shown with black arrow. The targeting arms aimed at the coding region are shown in red font, and the flanking region in the middle is shown in blue font (A). SfaN1: genotyping enzyme. Mutations in mpl<sup>smu3</sup>, the targeting region with 8-bp deletion (gray box) and 48-bp insertion (red box). The same sequence underlined with yellow. Frameshift mutation of mpl creates premature stop codons indicated with asterisk (B). Mpl protein structures in WT and mutant. Red slashes indicate the premature stop of Mpl protein (C). (D) mpl<sup>Δ8,+48</sup> mutated transcripts generated in mpl<sup>−/−</sup> mutants. Primer pairs wt_FPa/wt_RP, mut_FPa/wt_RP and co_FPa/co_RP were utilized for detecting WT form, mpl<sup>Δ8,+48</sup> form and both forms, respectively. Quantitative RT-PCR examining the relative abundance of WT form (left), mpl<sup>Δ8,+48</sup> form (middle) and both forms (right) mpl transcript in 2 dpf WT (black columns) and mpl<sup>Δ8,+48</sup> mutants (grey columns).

Figure S2. HSCs, erythroid, lymphoid and myeloid lineages are not affected by mpl mutation. (A-D) Erythroid and lymphoid lineages are not affected in mpl<sup>smu3</sup> mutants. 0-Dianisidine staining at 5 dpf (A), WISH of hbael (B), hbbe1 (C, indicated by white arrows) and ragl (C, indicated by red arrows) expression in 4-dpf and 5-dpf WT (upper panels) and mpl<sup>smu3</sup> mutant (lower panels) embryos. (D) Myeloid lineage is
not affected in \(mpl^{\text{smu3}}\) mutants. The antibody staining of Lcp1 expression in 3-dpf WT siblings and \(mpl^{\text{smu3}}\) mutant embryos. Scale bars: 50 µm.

**Figure S3. Rescue of thrombocytopenia in \(mpl^{\text{smu3}}\) mutants by \(mpl\) overexpression.**

(A, B) Rescue of \(mpl^{\text{smu3}}\) mutants. Restoration of \(cd41^+\) cells (A) and thrombocytic gene expressions (B) by heat-shock induced \(mpl\) overexpression. Representative images (left panels) and quantification (right columns) of WISH indicated \(cd41^+\) cells in \(mpl^{\text{smu3}},\text{hsp- mpl-eGFP}\) mutants with (+hs) or without (-hs) heatshock. Statistical significance was determined by paired Student’s \(t\)-test, \(n \geq 10\) mean ± SD, *** \(p < 0.001\), Scale bars: 50 µm (A). Relative levels of thrombocytic genes in \(mpl^{\text{smu3}},\text{hsp- mpl-eGFP}\) with (+hs) or without (-hs) heatshock (B), statistical significance was determined by paired Student’s \(t\)-test, \(n = 10\) per group, data were combined from four biological replicates, mean ± SD, * \(p < 0.05\), ** \(p < 0.01\).

**Figure S4. Disruption of Tpo/Mpl/Jak2 mediated thrombocytopoiesis by \(mpl\) mutation.** (A) Loss of TPO responses in \(mpl^{\text{smu3}}\) mutant embryos. Expression of \(cd41\) upregulated in WT but not in \(mpl^{\text{smu3}}\) mutant by \(tpo\) mRNA injection. (B-D) Thrombocytopenia caused by the inhibition of Jak2 in WT (B and C) but was not exacerbated in \(mpl^{\text{smu3}}\) mutant (D). Decreased expression of thrombocytic genes upon TG101209 (JAK2 inhibitor) treatment (B) and \(jak2a\) morpholino injection (C). Effects of \(jak2a^{\text{as}}\)-MOs could be rescued by wild-type \(jak2a\) mRNA (C). Thrombocytopenia is not exacerbated by the inhibition of Jak2 by TG101209 or
knockdown of jak2a (D). (E) Thrombocytosis in jak2a<sup>V518F</sup> overexpressed embryos indicated by the increased expression of thrombocytic genes. (F) Partial rescue of thrombocytopenia in mpl<sup>smu3</sup> mutant embryos by jak2a<sup>V518F</sup> overexpression, indicated by the restored expression of thrombocytic genes but not HSC markers. Thrombocytic genes: cd41, mpl, gp9 and nfe; HSC markers: runx1 and cmyb. Statistical significance was determined by paired Student’s t-test, n = 10 per group, data were combined from four biological replicates, mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure S5. Comparison of Tg(mpl:eGFP)smu4 and Tg(cd41:eGFP). (A) Relative expression levels of thrombocytic genes (gp9, kif1b, lrrc32 and nfe2) in sorted GFP<sup>+</sup> cells of 4.5-dpf Tg(mpl:eGFP)smu4 and Tg(cd41:eGFP) larvae. Expression level was normalized with β-actin expression. Statistical significance was determined using a two sample Student’s t-test, n ≥ 200 per group, data were combined from four biological replicates, mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001. (B, C) FACS analysis of mpl:eGFP (B) or cd41:eGFP (C) positive single cells by imaging flow cytometer. FSC-A was directly proportional to cell size and SSC-A was indicative of cellular granularity. Gates: yellow denotes small cells, purple denotes medium cells, and pink denotes large cells. Lower panels show the representative DIC and fluorescent images of cells from each gate. (D) Smeared sorted mpl:eGFP<sup>+</sup> cells (left panels) and cd41:eGFP<sup>+</sup> cells (right panels) were stained with May-Grünwald/Giemsa. Numbers in box represent cell counts in total smeared cells. Different sizes of cells were grouped by diameter: Small (∼ < 5μm), Medium (∼ 5 - 7.5μm) and Large (> 7.5μm). Scale bars: 10 μm. (E) Quantification of mpl:eGFP<sup>+</sup> cells and
cd41:eGFP* cells in the AGM region of 48-hpf Tg(mpl:eGFP)smu4 or Tg(cd41:eGFP). Scale bars: 50 µm. Statistical significance was determined using a two sample Student’s t-test, n ≥ 20, mean ± SD, *** p < 0.001.
Supplementary Movie Legends

Movie S1. The thrombocytopenic defect in Tg(cd41:eGFP);mpl<sup>smu3</sup>. (Related to Figure 1B). Imaging of CHT region in 4-dpf Tg(cd41:eGFP);mpl<sup>smu3</sup> mutant embryo.

Movie S2. CHT localized and circulating thrombocytes in Tg(cd41:eGFP). (Related to Figure 1B). Imaging of CHT region in 4-dpf WT Tg(cd41:eGFP) sibling embryo.

Movie S3. Live images of 2.5-dpf Tg(mpl:eGFP)smu4 embryos. (Related to Figure 5B).

Movie S4. Live images of the CHT region in 5-dpf Tg(mpl:eGFP)smu4 embryos. (Related to Figure 5B).
### Supplementary Table 1

<table>
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<th>Gene</th>
<th>5' Primer</th>
<th>3' Primer</th>
<th>β-Actin 5' Primer</th>
<th>3' Primer</th>
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<td>3'- CACAACCCAATGGATCTGTG -3'</td>
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<td>3'- CTTGACTTTTCCGGAAAGTATCTG -3'</td>
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A

Left target sequence  
Spacer  
Right target sequence

5’- AGAAAGACTTCACTTGCTTTTGGGATGCACCTGCCTGAAAAGTCTCTTG - 3’
3’- TCTTCTGAAGTGAAAGAAACCTACGTGGACACTTTCCAGGATAC - 5’

B

WT

mpl

3’ - AGAAAGACTTCACTTGCTTTTGGGATGCACCTGCCTGAAAAGTCTCTTG - 5’

mpl

3’ - AGAAAGACTTCACTTGCTTTTGGGATGCACCTGCCTGAAAAGTCTCTTG - 5’

C

Fibronectin type 3 domain

WT

mpl

transmembrane domain

D

WT

mpl

target region

relative mp/level

0 50% 100% 150% 200%

WT product  mutant product  common product

WT  mpl

mutant product

common product

***  ***  n.s.
**Figure S2**

**A** O-dianisdine staining

- WT
- mp<sup>smu3</sup>

**B** WISH of *hbae1*

- WT
- mp<sup>smu3</sup>

**C** WISH of *rag1* + *hbbe1*

- WT
- mp<sup>smu3</sup>

**D** Lcp1 antibody staining

- WT
- mp<sup>smu3</sup>
Figure S3

A

WISH of cd41

mpl^m^{nu3} control

mpl^m^{nu3}; hsp-mpl-eGFP

3 dpf

B

3 dpf relative mRNA levels

**

mpl^m^{nu3}; hsp-mpl-eGFP – hs

mpl^m^{nu3}; hsp-mpl-eGFP + hs

mpl^m^{nu3}; hsp-mpl-eGFP

3 dpf cd41+ cell numbers

mpl^m^{nu3}; hsp-mpl-eGFP – hs

mpl^m^{nu3}; hsp-mpl-eGFP + hs

mpl^m^{nu3}; hsp-mpl-eGFP

WISH of cd41

mpl^m^{nu3} control

mpl^m^{nu3}; hsp-mpl-eGFP

3 dpf
Figure S4

A. TPO treatment

B. JAK2 inhibitor (TG101209) treatment

C. jaka MO knockdown and mRNA rescue

D. JAK2 inhibition and jaka knockdown

E. jaka^{V581F} overexpression

F. jaka^{V581F} overexpression
Figure S5

A

Relative mRNA levels

Tg(mpl:eGFP)smu4;WT

Tg(cd41:eGFP);WT

gp9  kif1b  lrrc32  nfe2

B

mpl:eGFP

Intensity, MC, SSC

Small  Medium  Large

38.6%  50.9%  9.35%

C

cd41:eGFP

Intensity, MC, SSC

Small  Medium  Large

13.3%  55.5%  29.9%

D

mpl:eGFP

Small  Medium  Large

285/667  338/667  44/667

10µm

cd41:eGFP

Small  Medium  Large

113/615  300/615  202/615

10µm

E

48 hpf AGM GFP+ cell numbers

***

cd41:eGFP+  mpl:eGFP+